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## EXAMPLE VI

### Phosphoantibody to the Substrate Consensus Sequence for Akt: RXRXXT*

The Akt protein kinase is an important regulator of cell survival and insulin signaling, but very few of its *in vivo* targets have been identifed. Studies with synthetic peptide substrates of Akt (D.R. Alessi et al. FEBS Lett. 399:333-338 (1996)) as well as the analysis of known Akt phosphorylation sites on GSK-3 (T.F. Franke *et al.* Cell 88:435-437 (1997)), Bad (M. Pap *et al.* J. Biol. Chem. 273:19929-19932 (1998); Datta et al. Cell 91:231-241 (1997)), FKHR Brunet et al. Cell 96:857-868 (1999)), and Caspase-9 (M.H. Cardone et al. Science 282:1318-1321 (1998)) indicate that Akt phosphorylates its substrates only at a serine or threonine in a conserved motif characterized by arginine at positions –5 and –3.

To study and discover new Akt targets, an antibody was developed that specifically recognizes the phosphorylated form of the Akt substrate consensus sequence RXRXXT*. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Thr* represents phospho-threonine: Cys-X-X-X-Arg-X-Arg-X-X-Thr*-X-X-X-X. (SEQ ID NO: 45) The synthetic phospho-peptide was conjugated KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected

was used, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Thr* represents phospho-threonine: Cys-X-X-X-X-X-Arg-Arg-X-Thr*X-X-X-X. The synthetic phospho-peptide was conjugated to KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

(SEQ ID NO:46)

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4ºC, then spun at 11,200rpm at 4ºC for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Protein A eluate was first incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. This resin was then drained and the flow-through then incubated with phospho-peptide resin. This column was drained, washed twice with PBS, phospho-specific antibody eluted with 0.1M Glycine, pH 2.7 and pooled fractions neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4ºC.

*et al.* EMBO J. 15:6541-6551 (1996)). RSK1 (Ser381) and the PKC's also contain this consensus site, phosphorylation of which is required for their activity (K.N. Dalby *et al.* J. Biol. Chem. 273:1496-1505 (1998); L.M. Keranen *et al.* Curr. Biol. 5:1395-1403 (1995)).

To help study signaling pathways regulated by phosphorylation at these key regulatory sites we developed an antibody that detects phospho-serine and phospho-threonine only when preceded by tyrosine, tryptophan or phenylalanine or when followed by phenylalanine. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Ser* or Thr* represents phospho-serine or phospho-threonine: X-X-X-X-F-X-X-F-[S*/T*]-[F/Y]-X-X-X-X-C. This synthetic phospho-peptide was conjugated to KLH and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen. (SEQ ID NO: 47)

Once rabbits stared to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4ºC, then spun at 11,200 rpm at 4ºC for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from

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# Phospho-Akt Substrate Antibody

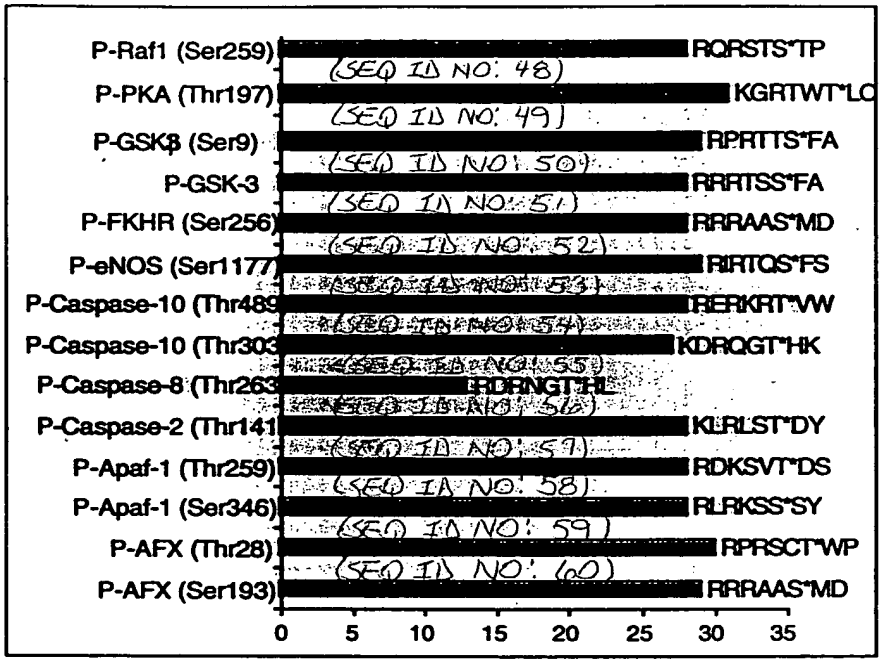


***Figure 6:*** *Signal to noise ratio of ELISA readings using Phospho-Akt Substrate Antibody with phospho-peptides of Akt substrates vs. non-phospho-peptides of Akt substrates.*

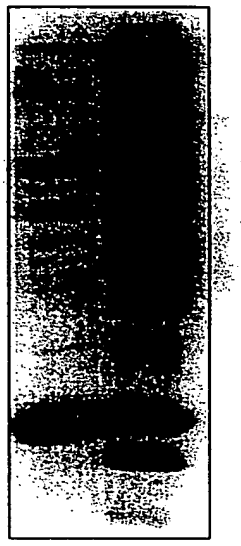


***Figure 7:*** *Western analysis of calyculin A-treated A431 cells using Phospho-Akt Substrate Antibody.*

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# Phospho-PKA Substrate Antibody

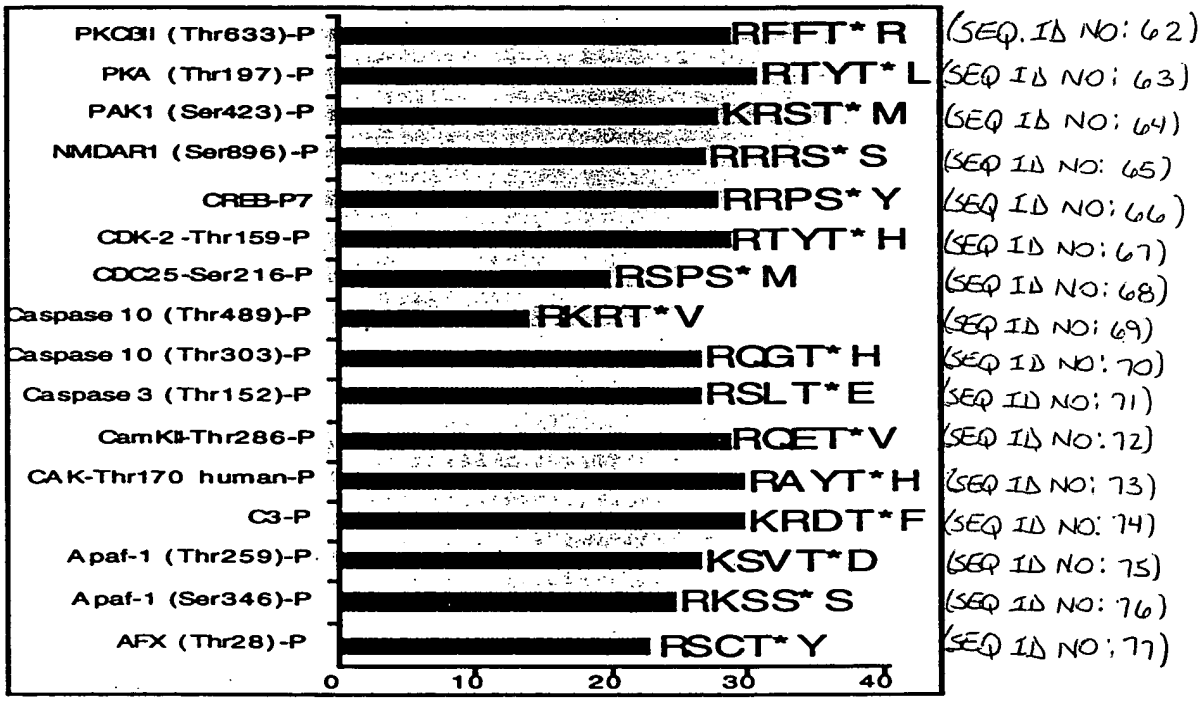


***Figure 8:*** *Signal to noise ratio of ELISA reading using phospho-PKA substrates antibody against peptides have Arginine or Lysine at -3 position.*

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Phospho-Serine/Threonine
Phenylalanine Antibody

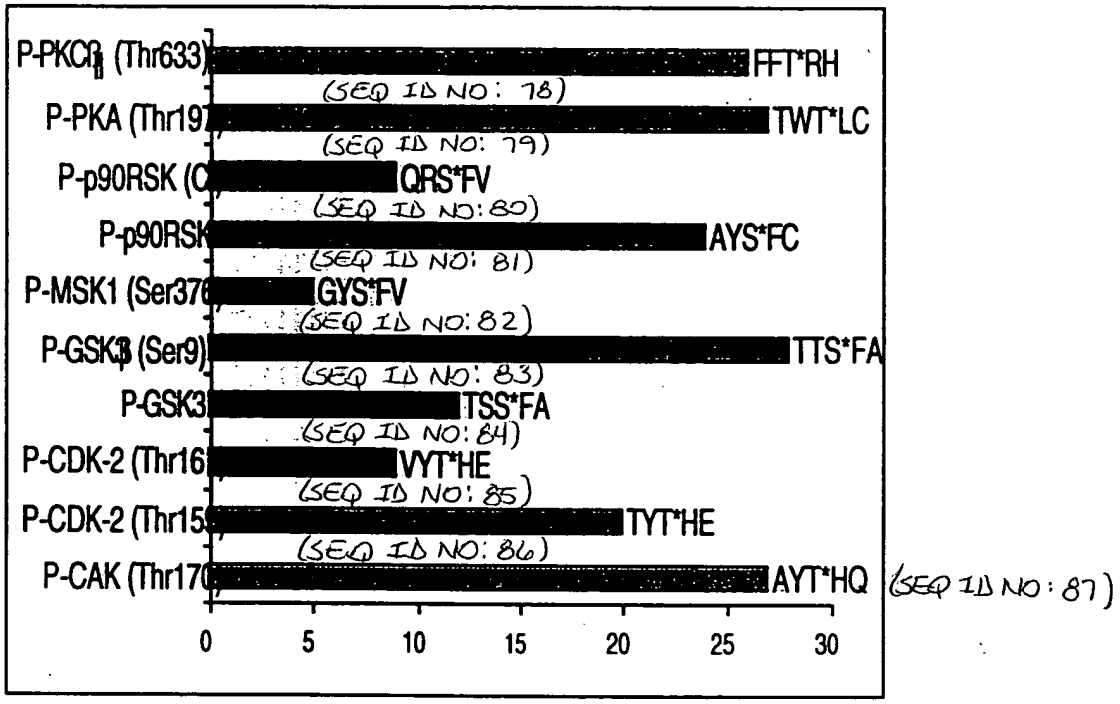
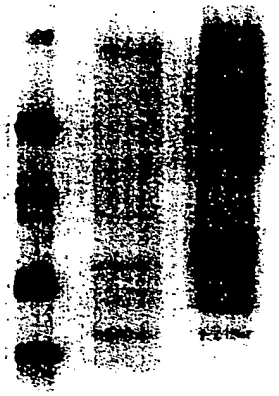



***Figure 11:*** *Signal to noise ratio of ELISA reading using phospho-Serine/threonine phenylalanine antibody aganist the peptides srounded by phenylalanine, tyrosine or tryptophan.*



\- +

calyculin A

***Figure 12:*** *Western analysis of calyculin A-treated A431 cells using phospho-Serine/phenylalanine substates antibody.*